

# Psoralen Promotes Myogenic Differentiation of Muscle Cells to Repair Fracture

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## ABSTRACT

Myogenic differentiation requires to be exactly explored for the effective treatment of fracture. The speed of healing is affected by skeletal muscle, linked to activation of specific myogenic transcription factors during the repair process. In previous study, we discovered that psoralen enhanced differentiation of osteoblast in primary mouse. In the current study, we show that psoralen stimulates myogenic differentiation through the secretion of factors to hone the quality of repair in fractured mice. 3-month old mice were treated with corn oil or psoralen followed by a tibial fracture surgery. Fractures were tested 7, 14, and 21 days respectively later by histology and images observation. Skeletal muscles including soleus muscle and posterior tibial muscle around the damaged bone were collected for quantitative real-time PCR, HE staining, as well as western blot. Daily treatment with psoralen at seven, fourteen days or twenty-one days improves protein or mRNA levels responsible for the whole myogenic differentiation process, makes the muscle fibers more tightly aligned, and promotes callus formation and development. This data shows that high levels of myogenic transcription factors in the process of fracture healing in mice foster the repair of damaged muscles, and indicates a pharmacological approach that targets myogenic differentiation to improve fracture repair. This also reflects the academic thought of "paying equal attention to both muscles and bones" in the prevention and treatment of fracture healing.

**Keywords:** Psoralen, Myogenic differentiation, Tibial fracture surgery, mRNA.

## Introduction

Fractures are a common disease in human. Despite the fact that the treatment of fracture has enhanced significantly in recent decades, a certain ratio of fractures remains complications and delayed healing [1]. According to recent reports, fracture with an incidence of 492,000 lead to about 77,000 hospitalizations and 825,000 physician visits in the United States in every year, including substantial both public and private health spending now as a health-care problem [2].

Although an excess of focus being paid to the mechanical environment, little emphasis has been given to the research of skeletal muscle during the process of fracture healing because of close relationship between two tissues except mechanical relationship [3]. Fracture repair is an intricate process in the terms of molecular and cellular levels, the regulation of which remains unknown [4]. Seemingly, when skeletal muscle is impaired, the final result of healing is obviously undermined. Moreover, according to Harry et al report, exploiting the model of open fracture in tibia show that when the uninjured muscle covered with fracture area, the space and quality of healing in fractured mice was better [5]. In addition, muscle stem cells play a crucial role in muscle microenvironment. In adult muscle, muscle satellite cells (SMSCs) normally keep quiescent, it nevertheless can exhibit the activity of SMSCs and differentiate into myoblasts after damage [6]. It is necessary, hence, to illuminate its possible mechanism in modulating myoblast differentiation of SMSCs. In the past, psoralen has been regarded as an effective drug for the treatment of bone-related diseases, including osteoporosis. Psoralen is an active ingredient stemming from psoralea corylifolia (Buguzhi), a medicine for bone diseases generally [7]. Based on our previous research, Psoralen had stimulatory effect on osteoblast differentiation via activating BMP signaling [8]. In present study, we show the specific mechanisms of psoralen on myogenic role with male mice, aged 3 months. The overall results indicate the contribution of psoralen to bone repair through myogenic differentiation.

## Experimental Procedures

### *Mice and treatment*

Animals' protocols were permitted by Experimental Animal Ethics Committee at Shanghai University of Traditional Chinese Medicine. C57BL6 mice were offered from Animal Room in Shanghai University of Traditional Chinese Medicine and placed under a 12-hour light and dark cycle.

Psoralen was obtained from Chengdu Herbpurify Biotechnology Co., Ltd. and the purity of psoralen is more than 99%. It was dissolved in corn oil and then delivered by gavage (20mg/kg/d). The control group was treated with corn oil.

Mice were given to either psoralen or corn oil randomly. Treatment begun with the day of surgery and progressed at duration of 7, 14 and 21 days. Doses were chosen on the basis of our prior studies.

### *Fracture generation*

Tibial fracture in mice aged 3 months with stabilization was used for studying fracture healing. In brief, mice were shaved in the surgical area behind anesthesia. The incision was made in the area of tendon, and small hole was drilled. Fixation was realized with an appropriate pin, and then diaphyseal tissues in tibia were transected with scissors. Finally, the incision was closed using sutures.

### *X-ray and $\mu$ CT*

After sacrifice at postoperative 7, 14, or 21 days separately, the surgical tibia in murine were examined. Next specimens were fixed and then placed in 75% ethanol. Needles in the tibia were cleared before using  $\mu$ CT. The outcome of fracture healing was analysed with a Viva CT 80 (Scanco, Brüttisellen, Switzerland).

### *Histology*

After sacrifice, soleus muscle and tibialis posterior of all mice was isolated carefully. All solus muscle was dehydrated, and then embedded into paraffin. Pathological changes of solus muscle were observed in HE staining. Tibialis posterior of all mice was prepared for protein and RNA analysis.

### *RNA extraction and real-time quantitative PCR (qPCR) of muscle*

All RNA was obtained from musculi tibialis posterior tissues using Tissue RNA Purification Kit PLUS (HiFunBio, China). The generation of cDNA for mRNA and the performance of RT-PCR reaction were according to 4 $\times$  EZscript Reverse Transcription Mix II (HiFunBio, China). Sangon Biotech Co., Ltd. (Shanghai, China) designed and provides the primer sequences, which was as follows:

GAPDH (forward: 5'-GCAGGAGTACGATGAGTCCG -3', reverse: 5'-ACGCAGCTCAGTAACAGTCC -3'), Myf5 (forward: 5'- GCAGCAGAAGAAACGTGTGAC-3', reverse: 5'-GGCTCAAACCTGGTCCCCAAA-3'), MyoD1 (forward: 5'CCGTGTTTCGACTCACCAGA-3', reverse: 5'-GTAGTAGGCGGTGTCGTAGC-3'), MEF2C (forward: 5'- ACTTGTGCAGAGGGATCACG-3', reverse: 5'-GGAACAGCTTGTTGGTGCTG-3'), and Myogenin (forward: 5'- GAGGAAGTCTGTGTCGGTG-3', reverse: 5'-CCACGATGGACGTAAGGGAG-3').

Relative gene expression was normalized with regard to GAPDH expression value.

### Western blot

All protein was secured from tibialis posterior tissues. The supernatant was obtained after removing cell debris. Protein concentration was determined by BCA protein Assay Kit. Then, proteins were divided be gels, transferred to polyvinylidene difluoride membranes, followed by incubation of appropriate antibodies. Bands were visualized with enhanced chemiluminescence followed by exposure to film. The expression level of protein was determined by calculating the intensity of bands in compare with control group and normalized to GAPDH using ImageJ software. The antibodies used were as follows: Anti-MyoD1 antibody (ab16148, abcam), Anti-MEF2C antibody (ab211493, abcam), Anti- Myf5 antibody (ab125078, abcam), Anti-Myogenin antibody (ab124800, abcam) and GAPDH Mouse monoclonal antibody (6004-1-Ig, proteintech).

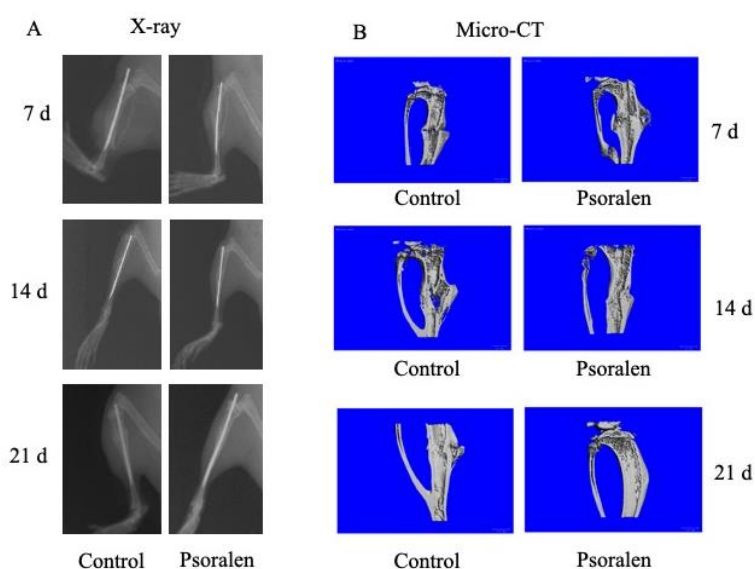
### Statistical analysis

Data above were expressed as means  $\pm$  SD followed by a two-tailed Student's t-test. Differences between the corn oil group and the psoralen group were determined by LSD-t test with  $p < 0.05$  regarded as significant difference.

### Results

#### *Psoralen accelerates ossification in the process of fracture repair*

In an effort to determine regulatory role of myogenic differentiation may enhance fracture recovery, 3 months old mice were administrated with psoralen after fracture surgery. Initially, the inflammatory period happens the first three days initiated by cell or tissue damage, but give way to cartilage after around 7 and 14 days. The cartilage is then displaced by bone. Therefore we chose appropriate time intervals to test the psoralen based on a characteristic course during fracture healing process. Mice in control group were provided with corn oil. At 7, 14, and 21 days after surgery, tibial specimens were examined histologically and radiographic imaging after sacrifice. First, we examined the fracture lines and bone connection between the two groups at 7, 14 and 21 days by X-ray.



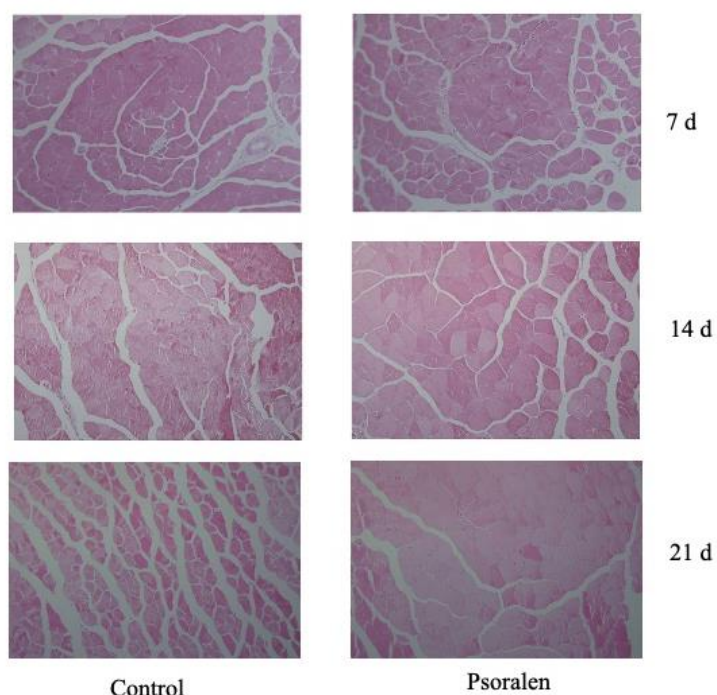
**Fig.1.** Psoralen treatment stimulates ossification during fracture healing in three-month-old male mice

(A) Tibial X-ray images of mice in the control group and the psoralen group, (B) Representative micro-CT images of tibial fracture at 7, 14 and 21 days.

As shown in Fig.1A, treatment with psoralen for 7, 14 and 21 days increased callus density and significantly blurred the fracture line at fracture site compared to controls. We also performed micro-CT reconstruction to visualize quantitative, and temporal composition of the fracture callus. As shown in Fig.1B, notably, Mice treated for 7 and 14 days after fracture with Psoralen showed less reticular callus and rather denser plate-like callus compared to carrier controls. Moreover, at 21 days after surgery, in psoralen group, no striking callus and fracture was healing completely, while in control group obvious callus existed.

### ***Psoralen boosted the repair of damaged muscle***

Next, we investigated the regulatory effect on the repair of musculus soleus in the fractured limb. Mice were treated for 7, 14 and 21 days after surgery, and compared to the control group at three time intervals after surgery respectively. As shown in Fig.2, histology indicated that arrangement of muscle fibers and number of muscle cell were changed after psoralen treatment. Specifically, no obvious changes were found in the psoralen group, compared with the controls at 7 days after a surgery. Hematoxylin & eosin staining, nevertheless, showed a tighter arrangement of muscle fibers and a relatively greater number of muscle cells in musculus soleus tissue with psoralen compared with the control group at 7 or 14 days after fracture. Due to possibly harmful effects of psoralen on which might change the process of repair, we examined the weight and signs of the mice of distress and no deleterious effect was observed in the mice, and no difference in weight between the psoralen group and control group mice.



**Fig.2.** HE staining of tibial tissues

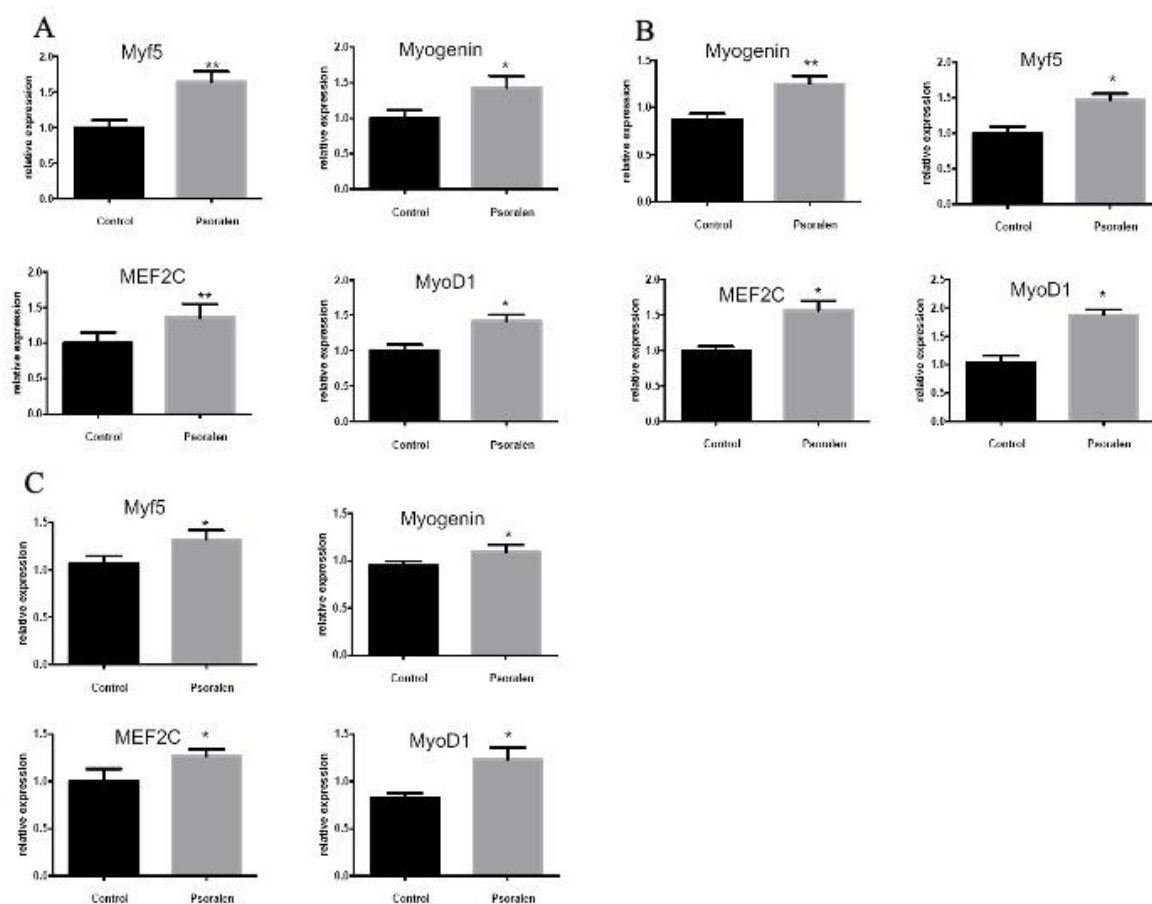
### ***Psoralen increased mRNA levels of myogenic transcription factors, and promoted myogenic differentiation***

Subjected to muscle damage after fracture, muscle satellite cells underwent a transition from QSCs (Quiescent satellite cells) to ASCs (activated satellite cells), normally regarded as muscle homeostasis, promoting their subsequent differentiation into myoblasts. The activation of satellite cells was affected partly via the induction of expression of myogenic factors (Myf5) and myogenic determination protein 1 (MyoD1). In addition, the

expression of myogenin (MyoG) and myocyte enhancer 2C (MEF2C) were involved in the differentiation of committed myoblasts especially during early period of satellite cell activation.

Recent studies have bear out an absolute requirement for these myogenic transcription factors, therefore for efficient recovery of muscle after damage that mediate satellite cell differentiation [9].

We thus examined mRNA expression of these myogenic transcription factors. The qPCR analysis was employed to test mRNA levels of these genes. Data was compared with controls. Treatment with psoralen at three time points (7, 14, and 21 days) notably increased the protein level of Myf5 (Fig.3A), MyoD1 (Fig.3B), MyoG (Fig.3C), and MEF2C (Fig.3D) in muscoli tibialis anterior.

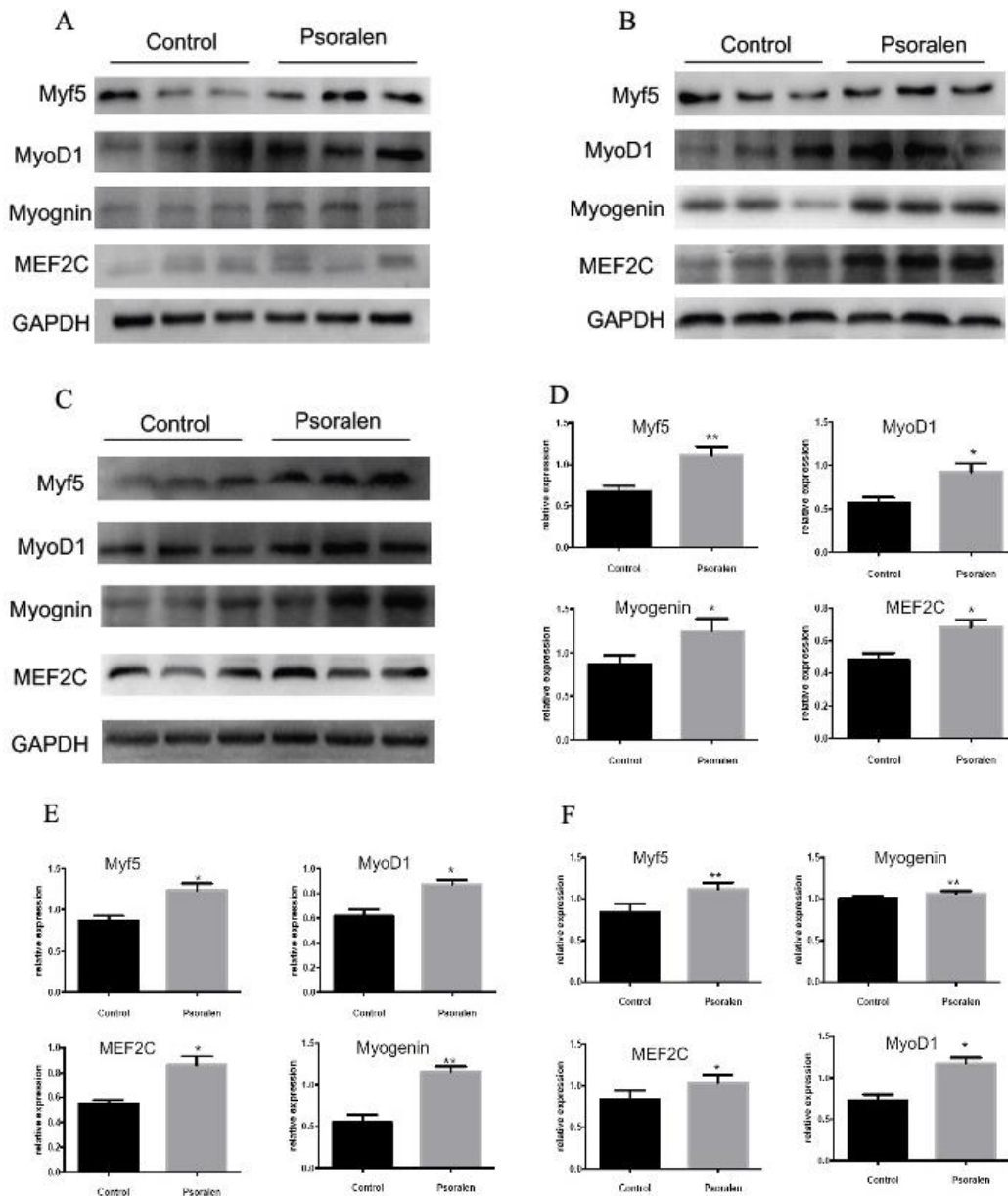


**Fig.3.** Psoralen promotes the mRNA levels of myogenic transcription factors in male mice. Three-month-old mice were treated with psoralen and corn oil for 7 (A), 14 (B) and 21 (C) days. The qPCR results showed that psoralen enhanced the mRNA levels of myogenic transcription factors in muscoli soleus at three time points generally. \* $p < 0.05$  when compared to the control group

### *Psoralen increased mRNA and protein levels of myogenic transcription factors, and promoted myogenic differentiation*

Consistent with the qPCR results, as shown in Fig.4, the protein levels of these four transcription factors after treatment with psoralen for 7, 14 and 21 days were also upregulated in comparison with controls. Taken together, these data indicated that psoralen was likely to exert its effects by mediating the level of specific myogenic transcription factors to the optimal level to stimulate differentiation of satellite muscle cells.





**Fig.4.** Psoralen modulates protein levels of myogenic transcription factors during fracture repair. Western blot analysis exhibited the expression of myogenic transcription factors treated with psoralen and corn oil for 7 (A), 14 (B) and 21 (C) days. Treatment of psoralen at 7 (D), 14 (E) and 21 (F) days increased the protein expression of Myf5, MyoD1, MyoG, and MEF2C, the specific myogenic transcription factors of myogenic differentiation. \* $p < 0.05$  compared with the control group

## Discussions

Fractures are frequent diseases in human. These injuries go on to successfully heal in most situations [10]. Nevertheless, non-union fracture is estimated to happen in over 5% of injuries, relying on a series of factors, including injury severity, and quality of fracture reduction [11]. Despite taking these factors into account, treatment may not lead to optimal healing, especially under circumstance of soft tissue trauma. In fact, the degree of soft tissue injury is closely related to fracture healing, whether it is open or closed shaft fracture from the clinical point of view [12]. On the one hand, fracture can lead to soft tissue edema, aggravate tissue ischemia and hypoxia, and

even muscle ischemic spasm, affecting fracture healing. On the other hand, because of intimate relationship between muscle and bone, muscle can provide vascular supply and cell population for the successful healing of fracture, and promote fracture healing [13]. Some studies have shown that the healing time of subperiosteal peeling is shorter than that of extra-periosteal peeling without muscle injury [14].

Furthermore, skeletal muscle is considered to secrete muscle factors (also known as myostatin), such as IL-8 seemingly stimulating angiogenesis, brain-derived neurotrophic factor (BDNF), as well as IL-15, a muscle factor decreasing obesity [15]. Quinn and his colleagues discovered that increased levels of IL-15 contributed to the increase of bone mineral content [16]. Thus, a wider understanding of bone-muscle biochemical crosstalk may provide novel insights into many other bone diseases.

Satellite cells play an indispensable role during the process of regeneration under the circumstance of injury in skeletal muscle [16]. They are maintained in a quiescent state under normal condition [17]. Paired box 7 (PAX7) instead of MyoD or MyoG usually express in quiescent QSCs [18]. When satellite cells receive signals from an injured situation, they are out of quiescent condition. They become activate and begin to proliferate [19].

Following proliferation, dividing satellite cells start to differentiation via upregulation of MyoG and downregulation or low-to undetectable levels of PAX7, finally retreating from cycle, fusing to repair muscle, and producing myoblasts. Recent studies demonstrate that MyoD —/— mutant murine show decreased muscle mass due to delayed myogenic differentiation. In addition, these MyoD—/—myoblasts do not ensure differentiation and fusion into myotubes [20]. These findings show that expression of MyoD function as a crucial role in myogenic differentiation. The expression of myogenin mostly relies on MyoD. Besides MyoD and myogenin, other factors, such as Myf5 and Mef2s comprised by myogenic regulatory factors (MRFs) have shown to have direct involvement in myogenic differentiation [9]. Among these factors, Myf5 and MyoD are primary transcription factors important for the modulatory gene expression program attributed to ASCs. Of note, main target genes induced by MRFs are specific structural and contractile genes with in muscle tissues, such as genes which encode actin or troponins. These genes expression is of great importance in skeletal muscle with respect to morphology, proper information, as well as function [21].

Satellite cells are regulated by several signaling pathways. First, emerging evidence indicates that Wnt/ $\beta$ -catenin pathway acts as a key role during regeneration in satellite cell function, despite of its controversial roles. Wnt signaling was reported to enhance myogenic involvement and terminal differentiation in myogenesis. Brack et al. [22] found that myoblast from cultured-single myofiber and in-vivo regenerating muscle response to Wnt signaling at a late period of differentiation alone, whereas inhibiting Wnt signaling generated the contradictory effect. Intriguingly, it seems that Notch signaling could counteract the impacts of Wnt/ $\beta$ -catenin signaling in the early stages by examining the levels of GSK3- $\beta$  which maintains usual levels of catenin in the cytoplasm by degradation of redundant catenin. Second, Notch signaling pathway also plays pivotal roles in skeletal regeneration. When the muscle is exposed to injury, Delta, a Notch ligand, is elevated in ASCs rapidly with the emerge of NICD, indicating the activation of Notch signaling, resulting in the expansion of proliferation of myoblasts [23].

Moreover, some molecules in sphingolipid signaling pathway has been taken for crucial molecules in terms of cell proliferation, death or senescence. Inhibiting the process of sphingomyelin-to-S1P demonstrated to promote

satellite cells into the cell cycle, improve muscle regeneration lessened the number of ASCs and impaired regeneration in muscle [24]. Nevertheless, further researches are requisite for comprehending the mechanisms that is how psoralen acts on these signaling pathways and pathologic microenvironment in fracture.

Psoralen has been extensively applied in some diseases because of pharmacological effects including its anti-inflammatory, anti-tumor activities and stimulating bone information [25]. It is known that psoralen is viewed as an attractive drug as it can induce anti-proliferation, and also differentiation in tumour cells [7]. Psoralen has been identified to stimulate osteoblast differentiating by activating BMP signaling pathway according to our previous study [8], thus possibly potential for therapeutic approaches of fracture. There is a saying that goes, "old drugs, new tricks." therefore we try to explore its new use in regard to fracture. This study highlights impacts of psoralen on tibial fracture that is promoting myogenic differentiation and enhancing fracture healing. The effects on muscle repair are also investigated. In this study, the degree of bone damage, healing in damaged skeletal muscle as well as the expression of protein connected with myogenic differentiation was tested. These above results suggest that psoralen may accelerate bone healing via acting on skeletal muscle in mice with fracture based on modulation of myogenic differentiation.

In the theory of traditional Chinese medicine, on the one hand, the quality of bone tissue is closely related to renal function, which is based on the theory that the kidney governs the bone. Therefore, traditional Chinese medicine for tonifying the kidney has been used to treat diseases such as fracture and joint injury for thousands of years. On the other hand, the spleen governs the muscles and transports water and grain essence, which is very important for the growth and development of musculoskeletal and physiological functions.

*Psoralea corylifolia* has a pungent and bitter taste, warm nature and two meridians of spleen and kidney. It is a commonly used medicine in the clinical treatment of osteoporosis, fracture and other diseases in traditional Chinese medicine. Psoralen is the main active ingredient. Our experiment illustrates that muscle, as the link between bone and bone, participates in the process of bone injury repair and remodeling through blood circulation, gene, molecular biology and other channels. This also reflects the academic thought of "paying equal attention to both muscles and bones" in the prevention and treatment of chronic muscle and bone diseases in traditional Chinese medicine.

In conclusion, we herein show for the first time that psoralen can promote muscle repair during bone fracture healing in 3-month old mice, which may be linked to its promoted effect on myogenic differentiation, contributing to faster pace of fracture repair. The protein level of myogenic differentiation factors, such as Myf5, MyoD1 and Myogenin, is differently elevated in the injured muscle after Psoralen treatment. Corresponding myogenic genes is upregulated for 7, 14 and 21 days after fracture compared to controls. Taken together, the effect of promoting myogenic differentiation could explain its enhanced muscle repair, providing protection against fracture. These novel applications of psoralen may be employed to facilitate the development of treatment of fracture.

## **Declarations**

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### Competing Interests Statement

*The authors declare no competing financial, professional and personal interests.*

### Consent for publication

*Authors declare that they consented for the publication of this research work.*

### Ethical Approval

*Animals' protocols were permitted by Experimental Animal Ethics Committee at Shanghai University of Traditional Chinese Medicine. C57BL6 mice were offered from Animal Room in Shanghai University of Traditional Chinese Medicine and placed under a 12-hour light and dark cycle.*

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